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Cloning of Acetylcholinesterase Gene in a Microbial Vector

Final Progress Report

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Acetylcholinesterase is the major site of action for several of the nerve gasses. It is therefore essential to elucidate the structure of the acetylcholinesterase molecule, in order to devise ways to protect against these poisons. This report describes attempts to obtain a cDNA clone encoding the acetylcholinesterase molecule. Such a clone would be useful because the primary amino acid sequence of the enzyme could be inferred from the cloned DNA sequence, and it could ultimately be used to produce sufficient quantities of the enzyme to determine its three

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20 ABSTRACT (cont.)

dimensional structure. The electric organ from Torpedo californica, which produces high levels of acetylcholinesterase, was used as the mRNA source for the recombinant DNA work. Identification of the mRNA encoding acetylcholinesterase (approximately .5% of the total mRNA) was accomplished by immunoprecipitation of radiolabeled in vitro translation products using an antiserum specific for the enzyme. The mRNA for the enzyme was characterized and enriched by fractionation on sucrose gradients. cDNA molecules were synthesized using both enriched mRNA and total RNA, and these were cloned in the plasmid vector, pBR322. These clones were screened by several methods including: differential colony hybridization with ^{32}P-cDNA probes, hybrid-selection translation, and DNA sequencing. The conclusion is made that cDNA sequences related to acetylcholinesterase mRNA have been successfully cloned. However, the unusually large size of the mRNA precluded obtaining the entire sequence, and from it the amino acid sequence, within the original contract period.

SUMMARY

Acetylcholinesterase is the major site of action for several of the nerve gasses. It is therefore essential to elucidate the structure of the acetylcholinesterase molecule, in order to devise ways to protect against these poisons. This report describes attempts to obtain a cDNA clone encoding the acetylcholinesterase molecule. Such a clone would be useful because the primary amino acid sequence of the enzyme could be inferred from the cloned DNA sequence, and it could ultimately be used to produce sufficient quantities of the enzyme to determine its three dimensional structure. The electric organ from Torpedo californica, which produces high levels of acetylcholinesterase, was used as the mRNA source for the recombinant DNA work. Identification of the mRNA encoding acetylcholinesterase (approximately .5% of the total mRNA) was accomplished by immunoprecipitation of radiolabeled in vitro translation products using an antiserum specific for the enzyme. The mRNA for the enzyme was characterized and enriched by fractionation on sucrose gradients. cDNA molecules were synthesized using both enriched mRNA and total mRNA, and these were cloned in the plasmid vector, pBR322. These clones were screened by several methods including: differential colony hybridization with ^{32}P-cDNA probes, hybrid-selection translation, and DNA sequencing. The conclusion is made that cDNA sequences related to acetylcholinesterase mRNA have been successfully cloned. However, the unusually large size of the mRNA precluded obtaining the entire sequence, and from it the amino acid sequence, within the original contract period.

FORWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication N. (NIH) 78-23, Revised 1978).

BACKGROUND AND APPROACH

Acetylcholinesterase plays a critical role in the regulation of cholinergic synapses by its activity to degrade the acetylcholine which remains after neurotransmission has occurred. Acetylcholinesterase exists in different forms, being either secreted, associated with the membrane, or associated with the extracellular matrix (1,2,3,4). The appearance of the enzyme in different forms is developmentally regulated (3), and sensitive to factors which influence synapse formation, synaptic transmission, and muscle activity (5). It is unknown how many genes for acetylcholinesterase exist. Koelle et al. (6) have postulated that acetylcholinesterase of the superior cervical ganglion may have pseudocholinesterase as a molecular precursor. On the basis of peptide mapping, amino acid compositions and antibody specificity (1,7) the existence of two different acetylcholinesterase molecules derived from separate mRNA's has been postulated to exist in the electric organ of Torpedo.

Acetylcholinesterase is the target of action of a number of compounds that block its activity either reversibly or irreversibly (8). Since these compounds could be used in wartime, it is critical to be able to block or ameliorate their actions. One approach to this problem is to obtain enough of the enzyme such that it can be crystallized and its three-dimensional structure determined. From the information derived it would be highly probable that a better means to block nerve gas action could be derived. Since the enzyme has low abundance and cannot be readily obtained in high yield, we have used recombinant DNA techniques as an alternative. Our approach to studies on acetylcholinesterase has been to isolate cDNA clones which are complementary to acetylcholinesterase-encoding mRNA, with the hope that the acetylcholinesterase protein sequence might be inferred from the DNA sequence. Although the human acetylcholinesterase structure may be the best to determine for medical applications, we chose to work on the enzyme from the electric organ of Torpedo californica, since acetylcholinesterase is more abundant in this tissue compared to human tissues, and since this enzyme has been characterized much more extensively.

We have been successful in isolating cDNA clones having properties related to acetylcholinesterase-encoding mRNA sequences; details of these studies are described below. The most interesting result of the studies so far is that these mRNA's are extremely large, about five times larger than the size necessary to accommodate the molecular weight of acetylcholinesterase. For this reason the elucidation of the acetylcholinesterase protein sequence must await more extensive DNA sequencing work.

MATERIALS AND METHODS

RNA was isolated from pulverized frozen electric organs of Torpedo californica (Pacific Biomarine, Venice CA) by high speed blending in guanidine thiocyanate and precipitation with LiCl as described (9). This method enabled us to obtain higher yields of more intact mRNA from the electric organ compared to centrifuging through a CsCl cushion. Poly(A) mRNA was purified over oligo dT cellulose (Collaborative Research)(10).

The poly (A) mRNA was further purified by fractionation on 13 to 25% sucrose gradients for 16 hours in a Beckman SW 40 rotor at 36,000 r.p.m. Identification of fractions enriched in acetylcholinesterase or acetylcholine receptor was performed by in vitro translation of the mRNA from each fraction in the presence of ^{35}S Methionine (Amersham) and immunoprecipitation using antiserum against the acetylcholinesterase or acetylcholine receptor (obtained from Drs. Palmer Taylor or Stanley Froehner, respectively), followed by examination of the products by SDS-PAGE (9,11).

cDNA libraries were constructed using either the unfractionated or the sucrose-gradient fractionated mRNA, by a procedure (12) which utilizes dGMP tailing of the first strand cDNA and second strand priming with oligo dCMP to enhance the production of full-length material. Size fractionation on a 10% acrylamide gel was also performed to remove the more common incomplete cDNAs.

Colony hybridization was used to screen the cDNA library made from fractionated RNA. Bacterial clones harboring cDNA-containing plasmids were grown as a library in 96-well tissue culture dishes (13), and replicate plated onto nitrocellulose filters (Schleicher and Schuell). The following sources were used to make single stranded ^{32}P-cDNA probes for comparison: a) unfractionated electric organ mRNA, b) fractionated electric organ mRNA, and c) nonpolyadenylated electric organ RNA.

The clones found to be positive in the colony hybridization analyses were tested by the hybrid-selection translation assay (14) using specific antiserum to Torpedo acetylcholinesterase for identification of the translation products.

RESULTS

We found that antiserum to Torpedo acetylcholinesterase, obtained from Dr. Palmer Taylor, could specifically immunoprecipitate 65,000 mol. wt. protein(s),the correct size for an unprocessed acetylcholinesterase subunit. This assay for translational activity became the basis for many of our subsequent analyses.

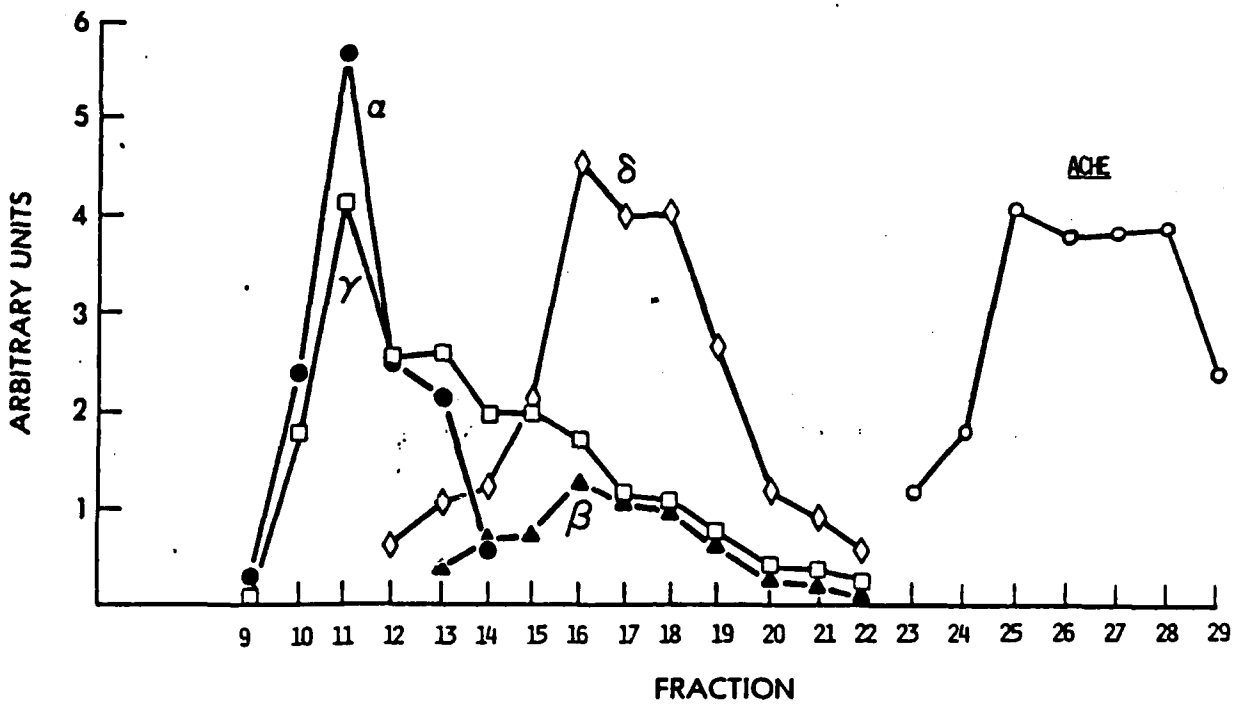

Figure 1.
Fractionation of Torpedo electric organ mRNA on 13%-25% sucrose gradients. Acetylcholinesterase mRNA activity was detected by in vitro translation in reticulocyte lysates, in the presence of ^{35}S-methionine. The products we then immunoprecipitated with both anti-acetylcholinesterase and anti-acetylcholine receptor antisera, and the precipitates were fractionated on 8.5% polyacrylamide gels and subjected to autoradiography. Shown is a densitometric tracing of the gel. Fraction 30 is at the bottom of the tube. α,β,γ and δ refer to the acetylcholine receptor subunits. ACHE = acetylcholinesterase.

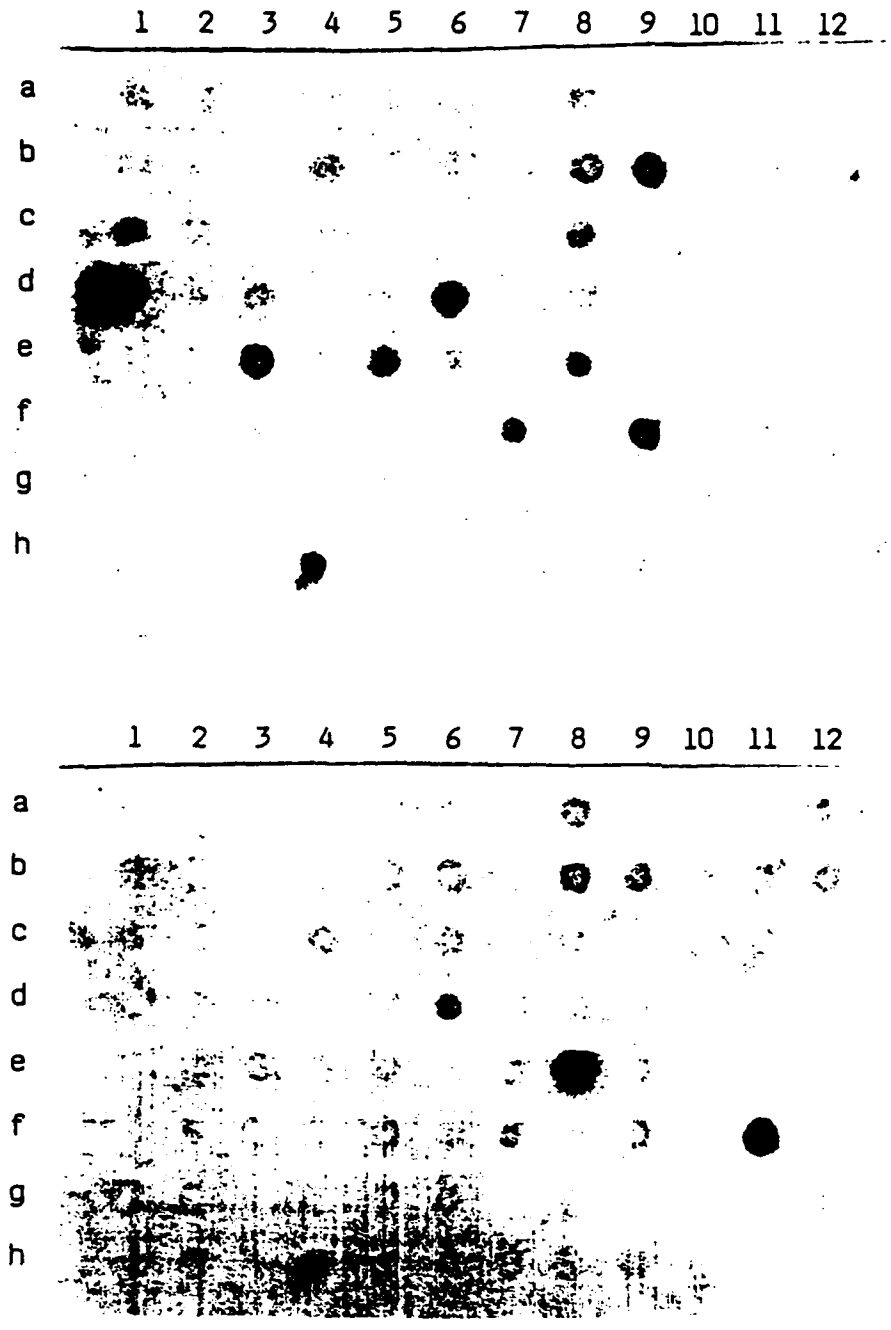

Figure 2. Replicate colony hybridization analysis of a portion of the clones containing plasmids with Torpedo electric organ cDNA inserts.

When the electric organ mRNA was fractionated on sucrose gradients, the profile of acetylcholinesterase translational activity gave a somewhat surprising result. The mRNA encoding the 65,000 mol. wt. acetylcholinesterase migrates very fast, a result implying that it is probably large (Figure 1). Using antiserum to the acetylcholine receptor we also assayed the fractions for receptor translational activities. After we had performed these analyses it was shown from the work of Noda et al. (15) that the mRNA for the 65,000 mol. wt. delta subunit of the receptor is about 6,000 bases long. Since the mRNA for the acetylcholinesterase migrates even faster, it is probably even larger.

Larger batches of electric organ RNA were fractionated on preparative sucrose gradients to yield small amounts of material for making cDNA libraries and cDNA probes. This material was approximately 5-fold enriched for acetylcholinesterase mRNA. We obtained about 280 cDNA clones (inserts from 200-1000 base-pairs) from the fractionated mRNA and over 3000 clones (inserts for 500-2000 base-pairs) from the unfractionated mRNA. Although the inserts were smaller, the cDNA clones made from fractionated RNA were screened first, since it was reasoned that 5-fold enrichment in acetylcholinesterase mRNA would help us obtain a first clone, which could then be used as a probe to obtain a longer clone from the bank made from unfractionated mRNA. This bank was demonstrated to be very useful, since it was possible to obtain several full-length cDNA clones encoding creatine kinase sequences (16).

The first screening procedure used was that of colony hybridization. Selection was based on the logic that clones containing acetylcholinesterase sequences should hybridize to a higher percentage of ^{32}P cDNA probe made from sucrose gradient-enriched mRNA than of probe made from unenriched mRNA. In addition, the correct clones should not hybridize to any of the ^{32}P cDNA made from unpolyadenylated RNA. As seen in Figure 2, we found colonies that bound more ^{32}P cDNA probe from enriched mRNA, compared to probe made from the unfractionated mRNA. These clones also showed no binding to probe made from non-polyadenylated RNA (data not shown). The clone seen at B9, later termed 2B9, was used for further analysis.

22 clones were positive in the colony hybridization analyses. At first, 13 of these were prepared for testing by the hybrid selection translation assay. One of these 13 gave a very weak positive signal. Attempts to characterize this clone were greatly hampered, since both of the PstI sites flanking the insert were destroyed in the cloning procedures, and no restriction enzymes could be found which cut within the insert. We finally were able to sequence this clone, only to find that it contained 40 dA residues, flanked by the dG/dC residues added for annealing to the vector.

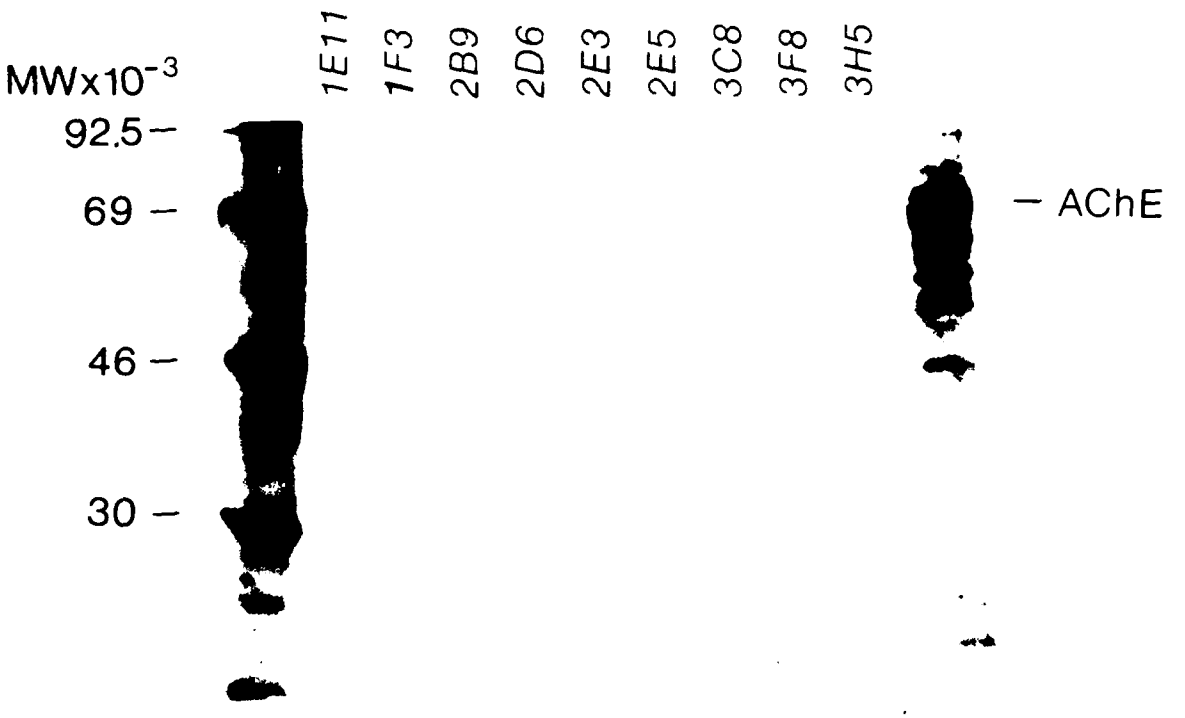

Figure 3.

Translation of acetylcholinesterase mRNA and immunoprecipitation of ^{32}S-labeled products, after selection by cloned plasmid DNAs. The molecular weight markers are on the left. Anti-acetylcholinesterase immunoprecipitation of total electric organ mRNA is on the right.

Although this initial result caused us to doubt the validity of the hybrid selection technique, we screened the remaining nine clones as well. This time, two clones (1F3 and 2B9) gave positive signals (Figure 3), both of which were more convincing than the earlier clone. We isolate the insert from clone 2B9 and labeled it with ^{32}P for use as a probe against all of our cDNA clones. Of the 22 clones picked by the colony hybridization experiment, only 1F3 and 2B9 hybridized to the 2B9 probe, indicating that 1F3 and 2B9 contain similar sequences. We view these results as strong evidence, although not proof, that we have successfully cloned DNA sequences related to the acetylcholinesterase mRNA.

A bank of 2600 clones made from unfractionated mRNA was also probed with the 2B9 ^{32}P-labeled insert and 13 were found to hybridize. These clones had inserts ranging in size from 500 to 1600 base-pairs.

DISCUSSION

An mRNA coding for the 65,000 mol. wt. acetylcholinesterase would most likely have a minimum length of 1900 bases. From the sucrose gradient data our mRNA probe probably has a length of more than 6000 bases. Since the longest clone obtained so far has only 1600 base-pairs, it would be necessary to isolate overlapping clones in order to derive to complete mRNA complement. It is possible that the clones obtained so far contain some of the acetylcholinesterase-encoding regions. We were encouraged by our progress up to that point, but because of the unforeseen peculiar length of the mRNA, the complete elucidation of the acetylcholinesterase sequence will require more time and effort.

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